Bacteriostatic Effect of Orally Administered Bovine Lactoferrin on Proliferation of *Clostridium* Species in the Gut of Mice Fed Bovine Milk

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When milk-fed mice were orally inoculated with *Clostridium ramosum* C1, this strain proliferated in the gut and became the dominant component of the fecal microflora. In this experimental model, bovine lactoferrin (bLF) administered with milk suppressed the proliferation of this strain in vivo and decreased the numbers of *C. ramosum* and other bacteria in the feces. This bacteriostatic effect of bLF was dependent on the concentration of bLF, the duration of feeding, and the administered dose of *C. ramosum* C1. Compared with bovine serum albumin, ovalbumin, bovine whey protein isolate, or bovine casein, only bLF showed this specific activity. A similar effect of bLF was observed after oral inoculation with *C. ramosum* JCM 1298, *C. paraputrificum* VPI 6372, or *C. perfringens* ATCC 13124. A hydrolysate prepared by digestion of bLF with porcine pepsin showed the same inhibitory effect on proliferation of *C. ramosum* in vivo as occurred with undigested bLF. These results indicate that ingested bLF can exert a bacteriostatic effect against clostridia in the gut even after it has been digested to some extent.

Lactoferrin, an iron-binding glycoprotein of the transferrin family, is present in most exocrine secretions of mammals. It has broad-spectrum antimicrobial properties and is thought to contribute to the host defense system active at mucosal surfaces and in colostrum and mature milk (4–6). Several studies have shown that breast-fed infants, who ingest substantial amounts of lactoferrin, have a lower incidence of gastrointestinal infections than formula-fed infants (7–9). However, despite a wealth of in vitro data, the antimicrobial role of lactoferrin in vivo is poorly understood (14).

Pepsin hydrolysates of bovine lactoferrin (bLF) or human lactoferrin (16) and an active peptide named lactoferricin isolated from such hydrolysates (1, 2) are known to display broad-spectrum antibacterial properties in vitro. We have recently reported that orally administered bLF exerts a bacteriostatic effect against intestinal members of the family *Enterobacteriaceae* in mice fed bovine milk, suggesting that lactoferrin present in mammalian milk may have the potential to protect infant animals from gastrointestinal disease (15). A hydrolysate of bLF digested with porcine pepsin (bLFH) showed the same inhibitory effect against intestinal *Enterobacteriaceae* as occurred with undigested bLF (15). From these results, it was postulated that bLF might influence the proliferation of other types of bacteria in the gut even after it has been digested. Presently, our interest is focused on whether lactoferrin exerts a bacteriostatic effect in vivo against clostridia present in the lower parts of the intestine.

To investigate the effect of ingested lactoferrin on intestinal clostridia, we developed a mouse model which dominantly harbors a detectable strain of *Clostridium* species in the gut. The model was established by oral administration of *Clostridium ramosum* C1 to mice fed bovine milk. Using this system, we have examined whether bLF or bLFH in milk fed to mice exerts a suppressive effect on in vivo proliferation of orally administered *Clostridium* species. Different species of *Clostridium* were tested in a similar manner to determine whether ingested bLF exerts a common effect against various intestinal clostridia.

MATERIALS AND METHODS

Preparation of bLF and bLFH. Native bLF and bLFH were prepared by the method described previously (15).

Bacteriological analysis. The numbers of clostridia in administered cell suspensions for animal experiments were assayed by using BL agar (13) (Eiken Chemical Co., Tokyo, Japan), a nonselective agar medium. The numbers of inoculated clostridia in feces were assayed by using BL agar and one of the following selective agar media: RCN agar for enumeration of *C. ramosum* C1, *C. ramosum* JCM 1298, and *C. innocuum* M601, CCNPOK agar for *C. paraputrificum* VPI 6372, CCNP agar for *C. butyricum* JCM 1391, and NC-NN agar for *C. perfringens* ATCC 13124. RCN agar (10) consists of EG agar (13), a nonselective medium for anaerobic bacteria, supplemented with 0.5 g of Tween 80 (Kanto Chemical Co., Tokyo, Japan), 5 mg of rifampin, 10 mg of colimycin, and 200 mg of neomycin sulfate per liter. CCNP agar consists of half-diluted (Gifu anaerobic medium) (GAM) agar (1.5% agar) containing 10 mg of colimycin, 50 mg of cycloserine, 100 mg of neomycin sulfate, and 10 mg of polymyxin B sulfate per liter. GAM agar, a nonselective agar medium for anaerobic bacteria, was purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Half-diluted GAM agar was prepared by diluting GAM agar with an equal volume of distilled water, and agar was added to a final concentration of 1.5%. CCNPOK agar consists of half-diluted GAM agar (1.5% agar) containing 20 mg of colimycin, 30 mg of cycloserine, 200 mg of neomycin sulfate, 20 mg of polymyxin B sulfate, 10 mg of oleandomycin phosphate, and 50 mg of kanamycin monosulfate per liter. CCNP and CCNPOK agars were prepared by adding 50 ml of sterile defibrinated horse blood (NIPPON BIO-SUPP. Center, Tokyo, Japan) per liter and filter-sterilized solutions of antibiotics at the above-specified final concentrations to half-diluted GAM agar after it was autoclaved and cooled. NC-NN agar was prepared by adding 5 mg of novobiocin and 10 mg of colimycin per liter to NN (neomycin Nagler) agar (13), a selective medium for lecithinase-positive clostridia. Compared with BL agar, the relative recoveries of the tested clostridia on these selective media were in the range of 90 to 100%. The rates of suppression of colony formation by fecal bacteria except the clostridia tested were less than 10^{-7} for RCN agar, 10^{-4} for CCNPOK and NC-NN agars, and 10^{-3} for CCNP agar. Rifampin, neomycin sulfate, cycloserine, polymyxin B sulfate, oleandomycin

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phosphate, kanamycin monosulfate, and novobiocin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Colimycin was purchased from Kayaku Co. (Tokyo, Japan).

Bacteriological analysis of the fecal microflora was performed by the methods of Mitsuoka et al. (12, 13). Approximately 0.2 g of feces was suspended at a dilution of 1/10 in an anaerobic prereduced diluent (12), and further serial 10-fold dilutions were prepared with oxygen-free CO_2 flowing. The oxygen-free CO_2 was prepared by passing CO_2 gas through a reduced copper column with an electric furnace (Sanshin Industry Co., Kanagawa, Japan). From appropriate dilutions, a 25- to 50-μl aliquot was spread onto nonselective and selective agar plates. All anaerobic agar plates were incubated in steel jars (Hirayama Manufacturing Corp., Tokyo, Japan) with steel wool covered by reduced copper under an atmosphere of CO_2 gas. After incubation for 1 to 3 days at 37°C, bacteria were identified by colony morphology and cellular features, Gram stain, spore formation, catalase activity, and aerobic and anaerobic growth characteristics. Clostridia isolated from feces were identified at the species level by the methods of Mitsuoka et al. (12) and Benno et al. (3). *Enterobacteriaceae* were identified by using the Enterotube II system (Becton Dickinson Overseas Inc., Tokyo, Japan) as described previously (15). Bacterial numbers were expressed as CFU per gram or milliliter of sample.

Bacterial strains and culture condition. *C. ramosum* C1 was isolated from feces of a female BALB/c specific-pathogen-free mouse obtained from Nihon SLC (Shizuoka, Japan). *C. paraputrificum* VPI 6372 was obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. *C. perfringens* ATCC 13124 was obtained from the American Type Culture Collection, Rockville, Md. *C. ramosum* JCM 1298 (ATCC 25582), *C. perfringens* C01, *C. butyricum* JCM 1391 (ATCC 19398), *C. clostridiiforme* U601, *C. innocuum* M601, *C. coccoides* JCM 1395 (ATCC 29236), and *C. difficile* JCM1296 (ATCC 9689) were obtained from the Institute of Physical and Chemical Research, Wako-City, Saitama, Japan. *Clostridium* strains were cultured in GAM broth, for 17 h at 37°C.

Determination of MICs. MICs of bLF and bLFH against the *Clostridium* strains to be administered were examined before the animal tests. Stock solutions of bLF and bLFH were prepared in distilled water and filter sterilized before addition to sterile culture medium. By using an inoculum of 10 μl of GAM broth culture (6 to 7 $\log_{10}$ CFU), each strain was cultured for 17 h at 37°C in a series of test tubes containing 5 ml of half-diluted GAM broth supplemented with 1% (vol/vol) antifoam DB-110N emulsion (Nacali Tesque Inc., Kyoto, Japan) and various defined concentrations of bLF or bLFH. Before cultivation, the medium was prereduced with oxygen-free CO_2 gas. The MIC was taken as the lowest concentration of bLF or bLFH that caused complete inhibition of bacterial growth.

Animals and diets. Three-week-old female BALB/c specific-pathogen-free mice were obtained from Nihon SLC. The mice were kept in a specific-pathogen-free room or occasionally in a vinyl isolator. They were initially fed a commercial pelleted diet (F-2; Funabashi Farms Co., Chiba, Japan) and tap water ad libitum for 7 days to allow them to become accustomed to the new environment. The mice (at 4 weeks of age) were then used in the experiments. The composition of tested diets and feeding schedules are described below. Other conditions of animal experiments were the same as those described previously (15).

In vivo proliferation of *C. ramosum*. The influence of milk feeding on in vivo proliferation of *C. ramosum* C1 was examined in four groups of five mice each. The influence of administered dose was also examined in this experiment. Each mouse was administered 0.1 ml of a cell suspension of *C. ramosum* C1 containing 7.0 $\log_{10}$ CFU (for two groups) or 5.0 $\log_{10}$ CFU (for the other two groups) applied directly into the stomach at day 0, using an oral catheter. After oral inoculation with *C. ramosum* C1, the two groups at each dose level were fed bovine milk or pellets ad libitum. Fresh feces were collected separately from each mouse at appropriate intervals during the experiment, and the number of *C. ramosum* in feces was assayed.

Influence of bLF on fecal microflora. The influence of orally administered bLF on the fecal microflora of mice which harbored *C. ramosum* as the dominant bacterium in feces was examined in nine mice. Each mouse was administered 7.0 $\log_{10}$ CFU of *C. ramosum* C1 at day 0 (after 7 days of preliminary feeding of pellets) and then fed bovine milk for 7 days. For the following 7 days (day 7 to 14), mice were fed milk containing 2% bLF.

Effects of bLF and bLFH on proliferation of *C. ramosum*. The influence of diets on the occurrence of *C. ramosum* C1 in feces, before and after oral inoculation, was examined in five groups of five mice each. Each mouse was administered 7.0 $\log_{10}$ CFU of *C. ramosum* C1. For 7 days before and 7 days after administration of *C. ramosum* C1, each group was fed one of five kinds of diets: milk only, milk followed after inoculation by milk containing 2% bLF, milk containing 2% bLF only, milk containing 2% bLFH only, or pellets only.

Effect of bLF concentration. The effect of bLF concentration on in vivo proliferation of *C. ramosum* C1 was examined in eight groups of five mice each. Two groups each were fed bovine milk containing bLF at a concentration of 0, 0.5, 1.0, or 2.0 % for 14 days. After 7 days of feeding, two groups given each concentration of bLF were administered 7.1 or 5.2 $\log_{10}$ CFU of *C. ramosum* C1.

Specificity of the bacteriostatic effect of bLF. The effects of various proteins (including bLF and bLFH) on in vivo proliferation of *C. ramosum* C1 were examined in seven groups of five mice each. Each group was fed bovine milk or milk containing one of the following proteins (15) at a concentration of 2%: bLF,

TABLE 1. Antibacterial activities of bLF and bLFH against various *Clostridium* species

Strain	Source	MIC (mg/ml)[a]	
		bLF	bLFH
C. ramosum C1	Mouse	>16	0.5
C. ramosum JCM 1298	Unknown	>16	1
C. paraputrificum VPI 6372	Human	>16	2
C. innocuum M601	Human	>16	2
C. perfringens ATCC 13124	Bovine	>16	>16
C. perfringens C01	Human	>16	>16
C. butyricum JCM 1391	Swine	>16	4
C. clostridiiforme U601	Human	8	0.25
C. coccoides JCM 1395	Mouse	4	0.25
C. difficile JCM 1296	Human	16	2

[a] bLF or bLFH was added to half-diluted GAM broth at final concentrations of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/ml.

bLFH, bovine serum albumin, ovalbumin, bovine casein, or bovine whey protein isolate. These diets were supplied for 14 days. After 7 days of feeding, each group was administered 5.0 $\log_{10}$ CFU of *C. ramosum* C1.

Effects of bLF on various *Clostridium* species. The effects of bLF on in vivo proliferation of other *Clostridium* species were examined. The *Clostridium* strains tested were *C. ramosum* JCM 1298, *C. paraputrificum* VPI 6372, *C. butyricum* JCM 1391, *C. innocuum* M601, and *C. perfringens* ATCC 13124. Mice were randomly divided into 10 groups of 5 or 10 mice each. Five of the ten groups were fed bovine milk, and the other five groups were fed milk containing 2% bLF for 14 days. After 7 days of feeding, one group fed milk and one group fed milk containing 2% bLF were administered 0.1 ml of a cell suspension prepared as described for *C. ramosum* C1.

Statistical analysis. Data obtained in animal experiments were expressed as the mean $\log_{10}$ CFU per gram ± standard deviation (SD_{n-1}) of samples. The data were analyzed statistically by one-way analysis of variance and the multiple-range test of Tukey-honest significant difference (HSD) and by Student's *t* test.

RESULTS

MICs of bLF and bLFH. Table 1 shows the MICs of bLF and bLFH against various *Clostridium* species isolated from different sources. The MIC of bLFH against each of the strains tested was less than that of undigested bLF, except for two strains of *C. perfringens*. Eight of the ten *Clostridium* strains tested were resistant to undigested bLF at concentrations up to 16 mg/ml.

In vivo proliferation of *C. ramosum*. Figure 1 shows the time course of in vivo proliferation of *C. ramosum* in mice. After oral inoculation with *C. ramosum* C1, the number of *C. ramosum* in feces of mice in the milk-fed groups increased greatly, to a level of 10 to 11 $\log_{10}$ CFU/g, within 7 days regardless of the dose of bacteria administered. The high numbers of *C. ramosum* in feces of the milk-fed groups remained at the same level up to day 14. *C. ramosum* was not detected (less than 2.3 $\log_{10}$ CFU/g) in the feces of mice of any group at day 0 (i.e., before inoculation with *C. ramosum* C1). There was no significant difference ($P < 0.05$) in fecal numbers on each day between the milk-fed groups administered 7.0 or 5.0 $\log_{10}$ CFU of *C. ramosum* C1. The total number of bacteria in the feces was around 11 $\log_{10}$ CFU/g, and *C. ramosum* was one of the most predominant species in the fecal microflora of mice fed bovine milk after day 7. On the other hand, a transient increase of *C. ramosum* was observed at day 1 in the group fed pellets, and the fecal number was dependent on the administered dose of bacteria. In the pellet-fed group administered a dose of 5.0 $\log_{10}$ CFU, *C. ramosum* was detected at a level of 3.1 $\log_{10}$ CFU/g in the feces of one of five mice at day 1. However, *C. ramosum* was not detected in the feces of the two groups fed pellets after day 2.

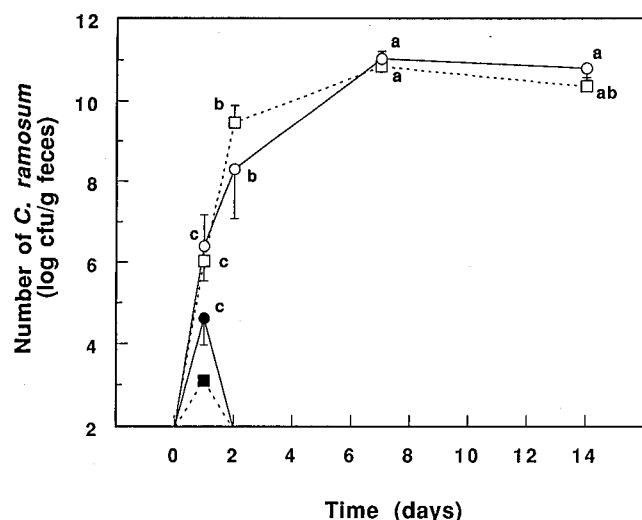The number of *C. ramosum* in feces increased rapidly just

FIG. 1. Proliferation of orally administered *C. ramosum* in mice. Two groups of mice were orally administered 0.1 ml of a cell suspension of *C. ramosum* C1 containing 7.0 $\log_{10}$ CFU at day 0 and fed bovine milk (○) or pellets and water (●) during the experiment. The other two groups were administered 5.0 $\log_{10}$ CFU at day 0 and fed bovine milk (□) or pellets and water (■). The values are expressed as mean $\log_{10}$ CFU per gram $\pm$ SD$_{n-1}$ ($n = 5$). Values of each group on different days and of three groups at day 1 with different letters are significantly different ($P < 0.05$) by the multiple-range test of Tukey-HSD. Values of two groups fed milk at days 2, 7, and 14 with different letters are significantly different ($P < 0.05$) by Student's *t* test.

after oral inoculation. Bacteria isolated from feces of each group on different days and identified as *C. ramosum* were confirmed to have the same microbiological properties as displayed by *C. ramosum* C1.

Influence of bLF on fecal microflora. Table 2 shows the changes of fecal microflora of mice before and after oral inoculation with *C. ramosum* C1. The most prevalent bacteria in the fecal microflora of mice fed pellets were lactobacilli and members of the family *Bacteroidaceae*. *C. ramosum* was not detected (less than 2.3 $\log_{10}$ CFU/g) in the feces of any mouse at day 0 (before inoculation with *C. ramosum* C1). The fecal numbers and incidence of other clostridia at day 0 were 7.7 $\pm$ 0.1 (mean $\log_{10}$ CFU/g $\pm$ SD$_{n-1}$) and three of nine, respectively. After inoculation with *C. ramosum* C1 followed by milk

feeding for 7 days (at day 7), *C. ramosum* became the most predominant bacterium. In addition, a significant increase in numbers of total bacteria, *Enterobacteriaceae*, streptococci, lactobacilli, bifidobacteria, and *C. ramosum* was observed at day 7 ($P < 0.05$). The incidence of bifidobacteria increased substantially at day 7. After feeding of milk containing 2% bLF to mice for another 7 days, the fecal numbers of total bacteria, *Enterobacteriaceae*, streptococci, lactobacilli, and *C. ramosum* decreased significantly at day 14 ($P < 0.05$). The number of bifidobacteria at day 14 remained at the same level as that at day 7. The numbers of staphylococci and *Bacteroidaceae* did not change during the experiment. The microflora of mice fed milk only for 14 days after oral inoculation with *C. ramosum* C1 showed no significant difference from that observed at day 7 (data not shown).

Effects of bLF and bLFH on proliferation of *C. ramosum*. The influence of diets before and after oral inoculation with *C. ramosum* C1 on in vivo proliferation of this strain was examined. The numbers of *C. ramosum* in feces of five groups fed different diets were compared 7 days after inoculation. Before inoculation, *C. ramosum* was not detected in the feces of any group. Seven days after inoculation, *C. ramosum* was detected in different numbers in the feces of milk-fed groups, including three groups fed bLF or bLFH. However, it was not detected in the feces of the group fed pellets. High numbers of *C. ramosum* in feces were obtained in groups fed the following diets for 7 days before and 7 days after inoculation, in order of decreasing frequency (mean $\log_{10}$ CFU per gram $\pm$ SD$_{n-1}$): milk only (10.1 $\pm$ 0.4), milk followed after inoculation by milk containing 2% bLF (8.1 $\pm$ 1.2), milk containing 2% bLFH only (7.3 $\pm$ 0.7), and milk containing 2% bLF only (5.5 $\pm$ 0.9). The numbers of *C. ramosum* in feces from the three groups fed 2% bLF or 2% bLFH were significantly less than those of the group fed milk only ($P < 0.05$). On the basis of this result, bLF or bLFH was administered for 7 days before and 7 days after inoculation in the following experiments.

Effects of bLF concentration. The effect of 2% bLF on in vivo proliferation of *C. ramosum* C1 administered in two doses to milk-fed mice was examined. The time course of proliferation of *C. ramosum* after inoculation is shown in Fig. 2. Before inoculation, *C. ramosum* was not detected in the feces of any groups. Proliferation of *C. ramosum* was suppressed significantly in the groups fed milk containing 2% bLF ($P < 0.01$) compared with the groups fed milk only. In the groups fed milk

TABLE 2. Influence of diets and administration of *C. ramosum* C1 on fecal microflora of mice[a]

Fecal microflora	Mean $\log_{10}$ CFU/g of feces $\pm$ SD$_{n-1}$ (frequency of occurrence [no. of mice yielding bacteria/9 examined])		
	Pellets (day 0)	BM (day 7)	BM + 2% bLF (day 14)
Total bacteria	9.8 $\pm$ 0.2[b]	11.0 $\pm$ 0.2[c]	10.4 $\pm$ 0.2[d]
Enterobacteriaceae	6.1 $\pm$ 0.4[b] (9)	8.5 $\pm$ 0.7[c] (9)	6.6 $\pm$ 0.6[b] (9)
Staphylococci	5.0 $\pm$ 0.7 (9)	4.7 $\pm$ 0.9 (9)	4.8 $\pm$ 0.6 (9)
Streptococci	6.3 $\pm$ 0.4[b] (9)	7.9 $\pm$ 0.3[c] (9)	6.6 $\pm$ 0.2[b] (9)
Lactobacilli	9.4 $\pm$ 0.5[b] (9)	9.8 $\pm$ 0.3[c] (9)	9.3 $\pm$ 0.2[b] (9)
Bacteroidaceae	9.4 $\pm$ 0.3 (9)	9.6 $\pm$ 0.2 (9)	9.7 $\pm$ 0.4 (9)
Bifidobacteria	7.6 (1)	10.0 $\pm$ 0.5 (9)	9.9 $\pm$ 0.4 (9)
Other anaerobic gram-positive rods	7.9 $\pm$ 0.3 (6)	8.4 $\pm$ 0.5 (2)	<7.6 (0)
Anaerobic gram-positive cocci	<7.6 (0)	8.9 (1)	<7.6 (0)
C. ramosum	<2.6 (0)	10.8 $\pm$ 0.4[b] (9)	9.8 $\pm$ 0.2[c] (9)

[a] Mice were administered *C. ramosum* C1 (7.0 $\log_{10}$ CFU/0.1 ml) at day 0 after 7 days of preliminary feeding of pellets and then fed bovine milk (BM) for the following 7 days and bovine milk containing 2% bLF for another 7 days.

[b–d] Values with different superscript letters for each type of bacteria are significantly different ($P < 0.05$) by the multiple-range test of Tukey-HSD and Student's *t* test for *C. ramosum*.
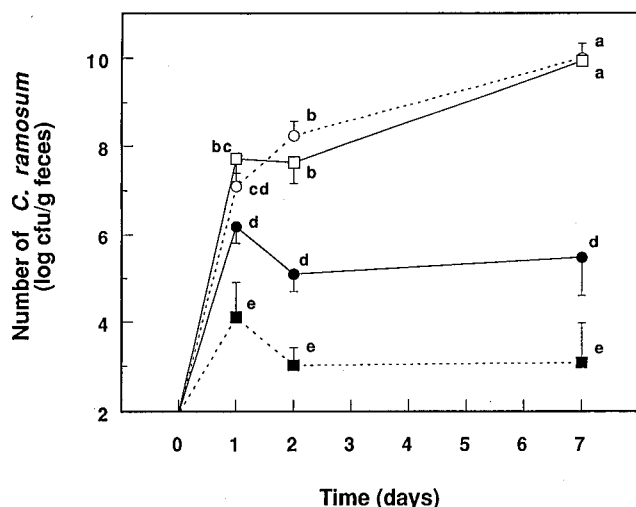
FIG. 2. Effects of 2% bLF on the proliferation of administered *C. ramosum* in mice fed bovine milk. Two groups of mice were orally administered 0.1 ml of a cell suspension of *C. ramosum* C1 containing 7.1 $\log_{10}$ CFU at day 0 and fed bovine milk (○) or milk containing 2% bLF (●) during the experiment. The other two groups were administered 5.2 $\log_{10}$ CFU at day 0 and fed bovine milk (□) or milk containing 2% bLF (■). Each group was fed the same diet for 7 days before and 7 days after inoculation. The values are expressed as mean $\log_{10}$ CFU per gram $\pm$ SD$_{n-1}$ ($n = 5$). Values of each group on different days and of four groups on each day with different letters are significantly different ($P < 0.01$) by the multiple-range test of Tukey-HSD.
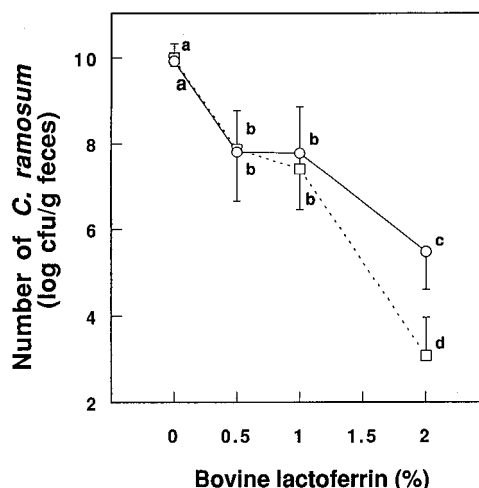

FIG. 3. Effects of bLF administered at different concentrations on the number of *C. ramosum* in feces of mice fed bovine milk. Each group of mice was fed bovine milk containing bovine lactoferrin at a concentration of 0, 0.5, 1.0, or 2.0% for 14 days. After 7 days of feeding, mice were orally administered 0.1 ml of a cell suspension of *C. ramosum* C1 containing 7.1 (○) or 5.2 (□) $\log_{10}$ CFU. The numbers of *C. ramosum* in feces 7 days after inoculation are shown. The values are expressed as mean $\log_{10}$ CFU per gram $\pm$ SD$_{n-1}$ ($n = 5$). Values of each group at different bLF concentrations with different letters are significantly different ($P < 0.05$) by the multiple-range test of Tukey-HSD. Values of two groups at the same concentration with different letters are significantly different ($P < 0.05$) by Student's *t* test.

containing 2% bLF, the numbers of *C. ramosum* in feces reached a maximum value at day 1 but decreased after day 2 to 1/10 of the maximum level. This transient increase appeared to be similar to that observed in the group fed pellets (Fig. 1). With the groups fed milk containing 2% bLF, the fecal numbers of *C. ramosum* in the group administered a dose of 5.2 $\log_{10}$ CFU were significantly less than observed in the group administered 7.1 $\log_{10}$ CFU during the period from days 1 to 7 ($P < 0.01$), indicating that the bacteriostatic effect of bLF was dependent on the administered dose of *C. ramosum* C1 (Fig. 2).

Figure 3 shows the effects of bLF administered at different concentrations on the numbers of *C. ramosum* in feces of milk-fed mice. The response of *C. ramosum* was dependent on the concentration of bLF administered. The numbers of fecal bacteria significantly decreased with bLF concentrations from 0.5 to 2% ($P < 0.05$). At 2% bLF, the fecal numbers in the group administered 5.2 $\log_{10}$ CFU of *C. ramosum* C1 decreased significantly ($P < 0.05$) compared with the group administered 7.1 $\log_{10}$ CFU.

Specificity of the bacteriostatic effect of bLF. The effects of five proteins (including bLF and bLFH) on the numbers of *C. ramosum* in feces of milk-fed mice are compared in Fig. 4. Among the proteins administered with bovine milk, only bLF showed this specific bacteriostatic effect against in vivo proliferation of *C. ramosum* in the gut. The numbers of *C. ramosum* in feces of the four groups fed milk containing bovine serum albumin, ovalbumin, whey protein isolate, or casein increased to the same level as that of the group fed milk only. On the other hand, the numbers of *C. ramosum* in feces of the two groups fed milk containing bLF or bLFH showed a significantly lower increase ($P < 0.001$) and remained at an extremely low level, less than 10^{-4} of that of the other groups.

Effects of bLF on various *Clostridium* species. Table 3 shows the bacteriostatic effects of bLF against various *Clostridium* species administered to milk-fed mice. The result obtained

with *C. ramosum* C1 from Fig. 3 is also included in this table to allow comparison with other species. Before inoculation, none of the *Clostridium* species to be administered was detected in the feces of any group. Feeding of milk containing 2% bLF resulted in a significant suppression of in vivo proliferation of three strains among the species tested, i.e., *C. ramosum* C1, *C.*
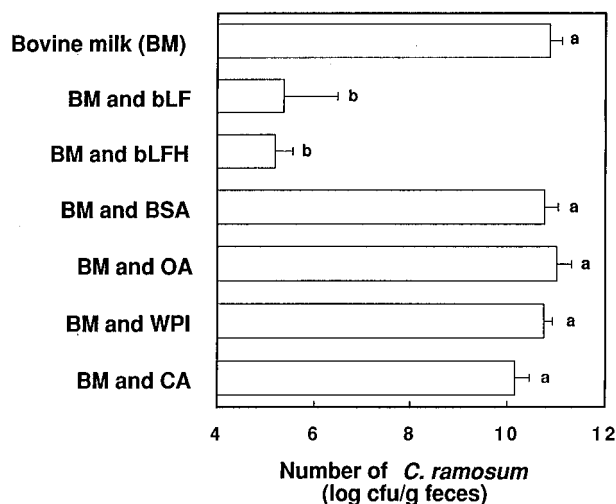

FIG. 4. Influence of administered proteins on the proliferation of *C. ramosum* in mice fed bovine milk. For 14 days, each group of mice was fed bovine milk or bovine milk supplemented with protein (bLF, bovine serum albumin [BSA], ovalbumin [OA], whey protein isolate [WPI], casein [CA], or bLFH) at a concentration of 2%. After 7 days of feeding, mice were orally administered 0.1 ml of a cell suspension of *C. ramosum* C1 containing 5.0 $\log_{10}$ CFU. The numbers of *C. ramosum* in feces 7 days after oral inoculation are shown. The values are expressed as mean $\log_{10}$ CFU per gram $\pm$ SD$_{n-1}$ ($n = 5$). Values with different letters are significantly different ($P < 0.001$) by the multiple-range test of Tukey-HSD.

TABLE 3. Effect of bLF on the proliferation of administered *Clostridium* spp. in mice fed bovine milk

Clostridium sp.	No. of administered cells[a] ($\log_{10}$ CFU/0.1 ml)	No. of *Clostridia*[b] as Mean $\log_{10}$ CFU/g of feces $\pm$ SD_{n-1} (frequency of occurrence [no. of mice yielding bacteria/no. examined])	
		Bovine milk	Bovine milk + 2% bLF
C. ramosum C1	5.2	10.0 ± 0.3 (5/5)	3.1 ± 0.9^c (5/5)
C. ramosum JCM 1298	5.7	10.6 ± 0.3 (5/5)	5.0 ± 1.3^c (5/5)
C. paraputrificum VPI 6372	7.1	7.8 ± 1.2 (5/5)	<4.6 (0/5)
C. butyricum JCM 1391	5.9	<6.6 (0/5)	<6.6 (0/5)
C. innocuum M601	5.6	6.9 ± 1.3 (5/5)	6.1 ± 1.1 (5/5)
C. perfringens ATCC 13124	6.7	4.4 ± 0.2 (2/10)	2.9 (1/10)

[a] Mice were orally administered 0.1 ml of cell suspension after 7 days of feeding.
[b] Number of clostridia detected in feces 7 days after oral inoculation.
[c] Significantly different ($P < 0.01$) by Student's *t* test compared with values for mice fed milk only.

ramosum JCM 1298, and *C. paraputrificum* VPI 6372 ($P < 0.01$). After oral inoculation with *C. paraputrificum* VPI 6372, this strain was not detected in feces of mice fed milk containing 2% bLF. In the case of mice fed milk only, the fecal numbers of administered *C. ramosum* JCM 1298 increased greatly to the same level as observed with *C. ramosum* C1. In the case of milk-fed mice orally inoculated with *C. paraputrificum* VPI 6372 or *C. innocuum* M601, these bacteria were detected in feces in moderate numbers. No significant effect of administered bLF was observed in mice orally inoculated with *C. innocuum* M601 ($P < 0.05$). Compared with these strains, substantially lower numbers and incidences of *C. perfringens* were detected in feces of mice orally inoculated with *C. perfringens* ATCC 13124. *C. butyricum* was not detected in the feces of mice orally inoculated with *C. butyricum* JCM 1391, with or without administration of bLF. A tendency for the fecal numbers and incidence of *C. perfringens* to decrease was observed in mice fed milk containing 2% bLF. Detectable levels of *C. paraputrificum* VPI 6372 and *C. butyricum* JCM 1391 were different because different selective media were used.

DISCUSSION

When milk-fed mice were inoculated orally with at least 5 $\log_{10}$ CFU of *C. ramosum* C1, the numbers of these bacteria in feces increased rapidly to a level of 10 to 11 $\log_{10}$ CFU/g and remained at this high level. This observation suggests that the colon of milk-fed mice is much more readily colonized by *C. ramosum* C1 than the colon of mice fed pellets. There was no significant difference in body weight or milk intake of mice among the groups examined regardless of inoculation with the *Clostridium* strains tested.

The addition of bLF to milk resulted in a clear suppression of in vivo proliferation (Table 3; Fig. 2 to 4) and a significant decrease in the numbers of *C. ramosum* C1 in feces (Table 2). These observations provide evidence of a bacteriostatic effect of lactoferrin in vivo. After feeding of milk for 7 days to mice inoculated with *C. ramosum* C1, the numbers of *Enterobacteriaceae*, streptococci, lactobacilli, and bifidobacteria, as well as those of *C. ramosum*, in feces increased significantly (Table 2). Such a tendency for intestinal bacteria of mice to increase in response to milk feeding has been reported previously (11, 15). It is worth noting that feeding of milk containing 2% bLF resulted in a decrease in numbers of the bacteria mentioned above (except bifidobacteria) and total bacterial numbers in feces (Table 2). Administered bLF apparently suppressed the bacterial overgrowth in the gut that is caused by milk feeding.

The effect of bLFH was the same as that of undigested bLF in vivo (Fig. 4), whereas bLFH showed even stronger antibac-

terial activity than bLF in vitro (Table 1). In the case of mice fed bovine milk containing 2% bLF, the residue of bLF in the feces was estimated to be less than 0.3 mg/g of feces by an enzyme-linked immunosorbent assay method. Therefore, most of the ingested bLF appears to be digested to low-molecular-weight peptides during passage through the gastrointestinal tract. The results of this study suggest that the antimicrobial effect of bLF exerted in the gut may be due to some digestion products of bLF such as bLFH. To elucidate the mechanism of the in vivo effect of bLF, we intend to investigate in a further study the effects of bLF peptides, including glycopeptides generated by digestion of bLF with other proteases in addition to gastric pepsin.

With the clostridia tested in this study, administered bLF showed a bacteriostatic effect against each of the strains detected in feces except *C. innocuum* M601. The lack of effect of bLF against *C. innocuum* M601 in vivo is unexpected because the strain was sensitive to bLFH in vitro. The ability of clostridia to colonize the colon appears to be host dependent. The species tested are commonly isolated from infections in humans but differ in the ability to colonize the intestine of mice. Considering the bacteriostatic effect of bLF observed in this study, lactoferrin contained in mammalian milk may have the potential to protect infant animals against gastrointestinal infections by some species of clostridia. Further studies are required to ascertain whether ingested lactoferrin is effective in vivo against pathogenic clostridia.

REFERENCES

1. **Bellamy, W., M. Takase, H. Wakabayashi, K. Kawase, and M. Tomita.** 1992. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. J. Appl. Bacteriol. **73:**472–479.
2. **Bellamy, W., M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, and M. Tomita.** 1992. Identification of the bactericidal domain of lactoferrin. Biochim. Biophys. Acta **1121:**130–136.
3. **Benno, Y., K. Suzuki, K. Narisawa, W. R. Bruce, and T. Mitsuoka.** 1986. Comparison of the fecal microflora in rural Japanese and urban Canadians. Microbiol. Immunol. **30:**521–531.
4. **Brock, J. H.** 1980. Lactoferrin in human milk: its role in iron absorption and protection against enteric infection in newborn infants. Arch. Dis. Child. **55:**417–421.
5. **Bullen, J. J.** 1975. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. Postgrad. Med. J. **51:**67–70.
6. **Bullen, J. J.** 1981. The significance of iron in infection. Rev. Infect. Dis. **3:**1127–1138.
7. **France, G. L., D. J. Marmer, and R. W. Steel.** 1980. Breast feeding and *Salmonella* infection. Am. J. Dis. Child. **134:**147–152.
8. **Gunn, R. A., A. M. Kimball, R. A. Pollard, J. C. Feeley, and R. A. Feldman.** 1979. Bottle feeding as a risk factor for cholera in infants. Lancet **ii:**730–732.
9. **Howie, P. W., J. S. Forsyth, S. A. Ogston, A. Clark, and C. du V. Florey.** 1990. Protective effect of breast feeding against infection. Br. Med. J. **300:**11–16.
10. **Mitsuoka, T.** 1980. A color atlas of anaerobic bacteria, p. 326. Sobunsya, Tokyo.

11. **Mitsuoka, T., and C. Kaneuchi.** 1977. Ecology of the bifidobacteria. Am. J. Clin. Nutr. **30:**1799–1810.
12. **Mitsuoka, T., K. Ohno, Y. Benno, K. Suzuki, and K. Nanba.** 1976. Die Faekalflora bei Menschen. IV. Mitteilung: Vergleich des neu entwickelten Verfahrens mit dem bisherigen ublichen Verfahren zur Darmfloraanalyse. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **234:**219–233.
13. **Mitsuoka, T., T. Sega, and S. Yamamoto.** 1965. Eine verbesserte Methodik der qualitativen und quantitativen Analyse der Darmflora von Menschen und Tieren. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1

Orig. Reihe A **195:**455–469.
14. **Sanchez, L., M. Calvo, and J. H. Brock.** 1992. Biological role of lactoferrin. Arch. Dis. Child. **67:**657–661.
15. **Teraguchi, S., K. Ozawa, S. Yasuda, K. Shin, Y. Fukuwatari, and S. Shimamura.** 1994. The bacteriostatic effects of orally administered bovine lactoferrin on intestinal *Enterobacteriaceae* of SPF mice fed bovine milk. Biosci. Biotechnol. Biochem. **58:**482–487.
16. **Tomita, T., W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, and K. Kawase.** 1991. Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. J. Dairy Sci. **74:**4137–4142.